THE METABOLISM OF THE PENICILLIA IN RELATION TO PENICILLIN BIOSYNTHESIS

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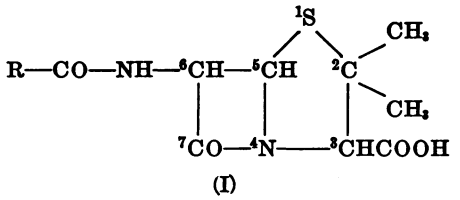
CONTENTS

INTRODUCTION

Penicillin, an outstanding example of selective toxicity among chemotherapeutic drugs, was reported by Fleming in 1929 to be present in the culture fluid of a species of penicillia (1). After the isolation in 1940 of relatively crude preparations, its extremely high antibacterial potency and negligible toxicity to humans were demonstrated (2). Subsequently, vast government-sponsored research programs, both in Great Britain and America, were commenced to develop methods of manufacture of the antibiotic, to undertake clinical testing and to investigate its chemical constitution and possible synthesis. Of this work, the chemical aspects have been recorded (3) and all aspects well reviewed (4).

Penicillin (I) was found not to be a single chemical entity, but rather a family of closely related compounds containing the β-lactam-thiazolidine ring system with differing side chains or R groups.



(I)

The identification of phenylacetyl-L-alanyl-D-valine as a hydrogenolysis product of benzylpenicillin (I, R = $C_6H_5 \cdot CH_2$—) indicates that carbon atom 6 of the β-lactam structure has the natural configuration and the asymmetric center at carbon atom 3 the unnatural configuration (5). The configuration of the asymmetric center at carbon atom 5 may be deduced from the X-ray structure. The numbering of the penicillin molecule follows conventional practice.

Although it has been known for many years that a large number of organic acids can be utilized by the mold to form the acyl side-chain of the penicillin molecule, the compounds and reaction sequences utilized in the formation of the β-lactam-thiazolidine ring system are still to a large extent unknown. In recent years there has been a renewed interest in the biosynthesis of this unique ring system, due partly to the possibility that such knowledge might result in increased yields of the antibiotic from fermentations and also to an interest in unusual intermediates and reactions. At the same time research on precursors of new R group penicillins has continued and has resulted in the discovery of phenoxymethyl-penicillin (R = $C_6H_5OCH_2 \cdot CO$—) which, in contrast to other penicillins, is more stable in acid solution (6).

In the course of these investigations much information concerning the occurrence of penicillin-producing strains and their metabolism has been reported; it is the purpose of this review to correlate these results (especially the more recent) with reference to the biosynthesis of penicillin.

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TABLE 1

Comparison of penicillin production by mutants of Penicillium chrysogenum NRRL 1951

Strain	Strain Origin	Penicillin Yield*	Reference
		units/ml.	
NRRL 1951	Original isolate	40–50	9
NRRL 1951-B	Spontaneous sector mutant of 1951	60–70	9
NRRL 1951-B25	Single spore isolate from 1951-B	245	10
X1612	X-ray mutant of 1951-B25	558	10
Q176	UV light mutant of X1612	904	10

* On similar media in shaken flasks.

THE DEVELOPMENT OF POTENT PENICILLIN-PRODUCING STRAINS

The property of penicillin production by fungi is not confined to a few strains, but is widely distributed among the penicillia and also occurs in the aspergilli (4). Entirely different groups of fungi, such as the pathogenic fungus *Trichophyton mentagrophytes* (4) the thermophilic fungus *Malbranchea pulchella* (4) and the fungus *Cephalosporium* (7) also produce penicillin-like substances. Nevertheless, this rather characteristic property is mainly associated with the *Penicillium notatum-chrysogenum* group. Of 241 members of this group isolated from soil and food samples from all over the world, only 24 failed to produce penicillin in measurable amounts. The remainder, however, varied markedly in their antibiotic-producing properties under identical conditions (8). The selection from natural sources of the higher yielding strains and their subsequent development has been a factor of great importance to the production of penicillin.

At first strains suitable for penicillin production by surface culture were developed by a series of monospore isolations of natural mutants and strains suitable for submerged fermentation were then sought. An excellent example of strain improvement by the selection of natural and induced mutants is furnished by the history of the development of *Penicillium chrysogenum* NRRL 1951. Discovered initially by surface culture methods, it proved to be a more efficient penicillin producer in submerged culture. Descendants of this strain are used today throughout the world for commercial production of the antibiotic. Details of the development of this strain are summarized in table 1. Under more favorable environmental conditions, the yield from Q176 has been vastly improved, for example 1550 units/ml was reported in 1946 (11) and 2000 units/ml in 1950 (12).

Strain Q176, however, suffers from the disadvantage that it produces a yellow extracellular pigment difficult to remove from penicillin by reason of its similar chemical properties. The discovery of a pigmentless mutant derived from Q176 initiated a further strain development program. Although penicillin yields of the mutant were inferior to those of its parent, it was superior to any other pigmentless strain encountered (13, 14). The process of strain selection from Q176 is summarized in figure 1. None of these mutant strains produced the undesirable pigment and under the same testing conditions (reciprocating shaken flasks with the precursor added at zero time) yielded more penicillin than the original parent Q176. For example, strain 47-911 produced about the same, 48-701 about 2.3 times and 49-

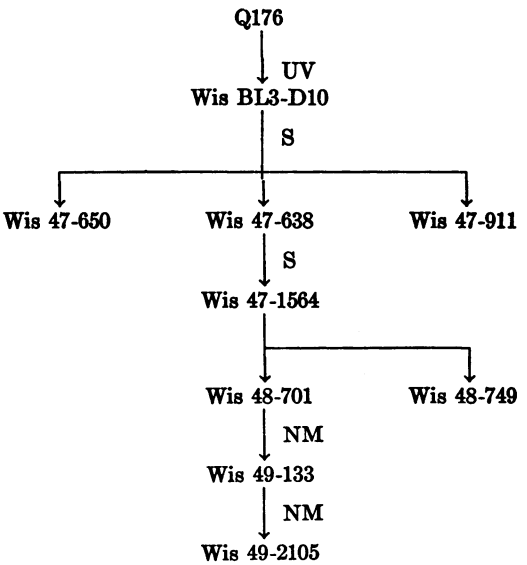


Figure 1. Genealogical derivation of pigmentless mutant strains derived from strain Q176. S = Selection, without treatment. UV = Selection following ultraviolet irradiation. NM = Selection following treatment with nitrogen mustard [methyl bis(β-chloroethyl)amine].

From the paper by Anderson *et al.* (13).

2105 about 3.7 times as much penicillin as Q176. However, under different fermentation conditions (stirred aerated jars) in a medium in which the maximum reported amounts of penicillin are obtained with Q176 these strains were decidedly inferior (14). Presumably, the higher metabolic conditions imposed may not suit these particular strains or the composition of the medium while optimal for Q176 may be suboptimal for the pigmentless mutants. It should be remembered that these strains were superior to their parent in penicillin production under one particular set of test fermentation conditions and will not necessarily be superior under all conditions. This is one of the practical problems involved in any search for valuable mutants, since a valid comparison of the inherent abilities of a number of different strains to synthesize penicillin can only be made when each is grown under its own optimum conditions. Such information is not yet available for these pigmentless strains. The primary object of these mutation programs was to produce high-yielding penicillin strains and in this respect marked success has been achieved. Of the more fundamental aspects, why a strain produces penicillin or why some mutants produce more penicillin than their parent, almost nothing is known.

Many metabolic pathways have been elucidated by the use of induced mutants of *Neurospora* which are more nutritionally exacting than the parent strain (15). On the basis of this classical work, a study of induced mutants of the *Penicillium notatum-chrysogenum* group was undertaken (16). Because the penicillia are imperfect fungi, crossing and genetic transmission as a proof of a single gene mutation is not possible. However, by analogy with the neurospora, there is little doubt of the genetic nature of the changes observed in such mutant strains. A total of 85,595 single spore isolates were tested, yielding 398 strains each deficient in the ability to synthesize some component of its cell material. Unfortunately, no significant correlation with penicillin biosynthesis could be observed. Further studies were made on non-penicillin producing mutants obtained from strains which gave good yields of penicillin (17). It was hoped that penicillin intermediates accumulating in the medium of a fermentation of any one of these mutants could be converted into penicillin by another such mutant, but no success was achieved. These non-penicillin producing strains were similar in their nutritional requirements to the original parent, which may be explained on the basis that a genetic block in penicillin synthesis does not result in an accessory growth factor requirement because penicillin is not necessary for normal metabolism or growth. Finally, none of a large variety of possible precursors or degradation products of penicillin induced penicillin formation by these mutants.

There is, however, one example of changes in penicillin formation which may be attributed to differences in the enzymatic make-up of parent and mutant strains. Thus, from an original soil isolate a strain was developed (NRRL 1954A), which produced about 15 per cent of the total penicillin as *p*-hydroxybenzylpenicillin on a corn-steep liquor medium in submerged culture. Conidia of this strain were irradiated with UV light and 50 monospore isolates were examined. One of these mutants under the same cultural conditions consistently gave 50 per cent of the total yield as *p*-hydroxybenzylpenicillin as judged by differential biological assays. This was equivalent to 65 to 70 per cent of the total yield on a weight basis. In a large scale fermentation, the latter estimate was confirmed by the isolation of the expected amount of this chloroform-insoluble penicillin (18).

ENVIRONMENTAL CONDITIONS IN RELATION TO PENICILLIN FORMATION

The development of optimal environmental conditions for antibiotic formation by various strains of the penicillia has been the subject of much study. For previous reviews the reader is referred to references (4), (14) and (19). In addition, the difficulties involved in providing adequate amounts of dissolved oxygen and nutrients to the cell surface have been discussed (12, 20).

Corn-steep liquor with lactose has for many years been the fermentation medium *par excellence* for the production of penicillin. Initially used for surface culture of the mold (21), it was subsequently utilized for the more successful submerged culture method, both in shaken flasks and stirred aerated jars. A by-product of the maize industry, corn-steep liquor is prepared by extracting maize with warm water. During the extraction or steeping period, bacterial fermentation takes place. To prevent putrefaction, sulfur dioxide is then added and the extract concentrated (22). Chemical analysis and fractionation

of this variable and complex concentrate (22, 23, 24) has not entirely explained its excellence as a medium for penicillin production. However, growth and penicillin production equal to that of a lactose corn-steep fermentation have been obtained recently with a medium composed essentially of glucose, inorganic salts and ammonia (25), indicating that the strain used in these experiments and probably other penicillia are not exacting in their nutritional requirements. Moreover, it seems that the excellence of the lactose corn-steep liquor medium is due not only to the presence of the appropriate nutrients, but also to their correct balance whereby the metabolism of the mold creates the correct environmental conditions for the subsequent production of penicillin. This viewpoint will be illustrated by outlining the chemical changes taking place in the medium and the metabolism of the mold during the fermentation.

It is generally agreed that three distinct metabolic phases can be distinguished in penicillin-producing cultures (26, 10, 12–14, 25), *viz.*, rapid growth followed by restricted growth with penicillin formation and finally autolysis. These phases will be discussed in that order.

Phase 1: Reactions connected with mycelial growth. In a lactose corn-steep liquor medium, glucose and lactate are metabolized at a maximum rate, but lactose is used very slowly. Organic and nitrate nitrogen are rapidly assimilated into the growing mycelium and the respiratory activity as shown by the Q_{O_2} is at a maximum. As growth proceeds, cation accumulation due to lactate utilization causes a slow increase of pH from the initial value of about 4–5. Towards the end of this phase, ammonia nitrogen accumulates in the medium and the pH rises to near neutrality (26). It has been suggested (13) that these events are related in the following manner. When all glucose and lactate have been consumed, there is an adaptation period before lactose utilization commences. To supply the energy demands of the mycelium over this period, amino acids in the medium are utilized as sources of carbon resulting in the accumulation of ammonia. Briefly, it appears that this complex medium contains sufficient rapidly oxidizable substrate to permit good mycelial growth, but subsequently, ammonia accumulates in the medium with a consequent elevation of the pH to neutrality. The same sequence of events has been achieved by the correct balance between glucose and ammonium acetate in a chemically-defined medium, based on the observation that the utilization of acetate ions proceeded at a much faster rate than that of ammonium ions during glucose utilization by the mold at pH 6.2 (27). Similarly, for good penicillin formation the mold can be grown on a limiting amount of glucose with inorganic salts at pH 5, the pH being raised to neutrality with gaseous ammonia when the carbohydrate is exhausted. However, where the pH is controlled directly and not by a balance of medium constituents, the pH of the growth phase does not appear to be of importance. Thus a fermentation conducted entirely in the pH range 7–7.5, actually produced more penicillin than one with an initial growth phase at pH 5.5 (25).

The amount of mycelium produced in a fermentation does not appear to be correlated with the amount of penicillin produced. For example, strains 49-133 and 49-2105 produced only about 50 per cent as much mycelium as strain 47-1380 and 48-701 under identical conditions, but gave significantly higher yields of penicillin (13).

Phase 2: Penicillin production. This phase is connected with the utilization of lactose and ammonia nitrogen. The rate of mycelial growth is low, respiratory activity is reduced and the pH remains stationary or rises slowly. A pH of near neutrality has been found most suitable for this phase, both with corn-steep liquor (12) and chemically-defined medium in shake flasks (27, 28) and stirred aerated jars (25).

The function of lactose in this phase has been generally accepted as that of a slowly utilized carbon source which permits the prolonged slow development of mycelium at a neutral pH. Under these conditions penicillin is formed, and the more this phase can be prolonged, the greater the yield of penicillin. For example, when lactose is replaced by glucose (a rapidly utilized compound) in a corn-steep liquor culture, the phase of penicillin formation is short, and little penicillin is produced. The rapid catabolism of glucose and lactate leads to the premature exhaustion of nutrients and autolysis sets in (29). However, lactose may be replaced by the addition of small amounts of glucose every 24 hours to a culture in the penicillin phase (14) and, even more successfully from the point of view of penicillin formation, by a continuous drip-feed of glucose, glucose feed rates of 0.03 to 0.07 per cent per hour giving

excellent penicillin yields (25). Below 0.03 per cent per hour insufficient nutrient is available for the mycelium, resulting in premature autolysis. Above a feed-rate of 0.07 per cent per hour, mold growth becomes very thick, and either the supply of air or nutrient to the cell surface becomes limiting, so that again there is autolysis. Between these extremes, represented by a tendency to starve and a tendency to grow, penicillin is produced at a high rate. Thus, a culture which was drip-fed glucose (0.042 per cent per hour) during the penicillin phase produced 1650 units of penicillin per ml at 140 hours. In contrast, a similar fermentation, to which lactose was added at the beginning of the culture utilized sugar at the rate of 0.03 per cent per hour, was complete in 100 hours and produced only 650 units of penicillin per ml. Both cultures produced about the same amount of mycelium as judged by nitrogen estimation. It seems likely, therefore, that the advantage of drip-fed glucose over lactose lies in its ability to prolong the phase of penicillin production by maintaining the mycelium in the appropriate metabolic state.

Other rapidly utilized sugars such as sucrose, starch, galactose, xylose and sorbose, also gave good results when added in small portions during the penicillin phase of a fermentation (14). Since glucose and galactose are both rapidly utilized, it seems possible that the slow metabolism of lactose may be occasioned by a slow enzymatic hydrolysis of the β-galactosidic linkage of lactose. These facts, coupled with the observed adaptation period before lactose utilization commenced in a lactose corn-steep liquor medium (13) (see above, under phase 1), could indicate an adaptive β-galactosidase formation by the mold. However, a similar delay has been observed in the utilization of drip-fed glucose in cultures grown in chemically-defined medium with an acid pH in the growth phase, although not apparently in cultures grown in the same medium at constant pH (7–7.5) over the whole culture period (25). In view of these observations, the validity of the adaptive β-galactosidase hypothesis may be questioned.

The utilization of ammonia during the penicillin-producing phase has already been mentioned. This assimilation is evidently a prerequisite of penicillin formation, for, in general, high ammonia levels in corn-steep liquor media are associated with low penicillin yields. It is not likely that the high ammonia concentration is the direct cause of the low penicillin yield, since the addition of ammonia to normal fermentations does not depress the yield of penicillin (26). Moreover, gaseous ammonia has been used successfully to control the pH (25), but it is not clear at what stages the additions were made.

Phase 3: Autolysis. In this phase, the small residual amount of carbohydrate is utilized, and the weight and nitrogen content of the mycelium decreases because of autolysis of the older cells. The consequent release of ammonia into the medium causes a slow rise of the pH to a value of 8–9. Since penicillin is unstable at this pH, the observed decrease in the concentration of penicillin in this phase may be explained on this basis.

The two types of culture from which most of the observations discussed here have been made are shaken-flasks and stirred aerated jars. One very obvious difference between these culture methods is the efficiency of aeration, *i.e.*, the gas exchange at the cell surface. The respiratory activities of the two types of mycelium have been compared (30), and it was concluded that the degree of aeration influences not only the quantity but also the kind of respiratory enzymes synthesized. However, the requirements for penicillin formation in these two types of culture are similar and may be summarized as follows:

1. A readily available source of nitrogen and carbon, the latter being required to supply energy as well as material for mycelial growth in phase 1, the amount of this carbon source being limited to prevent excessive growth of mycelium.

2. A slowly utilizable carbon source to maintain the energy requirements and allow only slow growth of the mycelium during phase 2. The type and method of supply of this source are critical for a good yield of the antibiotic.

3. The provision of a means of controlling the pH, either by an automatic device or by a balance of medium constituents, the pH being adjusted to a region suitable for rapid mycelial growth and subsequently to a region in which penicillin is produced at a rapid rate.

4. In a chemically-defined medium an adequate and balanced supply of inorganic salts is required. This subject has not been discussed here, but is dealt with elsewhere (31).

In conclusion, it must be emphasized that, although the foregoing information concerning the requirements of penicillin-producing fermen-

tations is of great practical value, the relation of the factors controlling penicillin production to the mechanism of biosynthesis remains at present unknown.

CERTAIN ASPECTS OF THE METABOLISM OF THE PENICILLIA

Supplementary to the general studies on the chemical and metabolic changes taking place during penicillin-producing fermentations many scattered observations exist on particular metabolic sequences and compounds which occur in both the culture medium and the mold mycelium. Although not all of these have a direct bearing on the problem of penicillin biosynthesis, they are of interest because the overall pattern so far discernible is similar to that found in other living organisms.

Sulfur Metabolism

The ability of fungi to utilize sulfate for growth and synthesis of organic sulfur compounds is almost universal, the penicillia being no exception. This fact has been used for the production of S^{35}-labeled penicillin from labeled inorganic sulfate added to culture media (32, 33).

The mechanism of the conversion of inorganic sulfur to organic sulfur by molds is, however, still obscure. It has been suggested that in the neurospora reduction follows the pathway sulfate → sulfite → thiosulfate → cysteine (34) and such a reduction sequence might also occur in the penicillia. Thus, from any one of these compounds as the sole source of sulfur similar yields of mycelium and penicillin were obtained from surface cultures of *Penicillium notatum*. Two mutants derived from this strain were, however, unable to utilize sulfate or sulfite, but grew and produced penicillin on the addition of thiosulfate or cysteine. No connection could be observed between the use of any particular sulfur source and penicillin formation (35). Similarly, with strain Wis 48-701 in a purified medium, the total amount of penicillin formed after 100 hours was the same whether the sole source of sulfur was inorganic sulfate, L-cysteine, DL-methionine, glutathione, cysteic acid, sodium taurocholate or choline sulfate (36).

It has been reported that 5-day-old mycelium of strain Wis 49-133 contains large amounts of "loosely-bound" methionine which was released by extraction with hot water and represented some 30 per cent of the sulfur originally present in the chemically defined culture medium (37). Working with the same strain grown in a different culture medium, De Flines (38) could not confirm this observation but suggested that this discrepancy might arise from different extraction techniques.

The medium at the end of a fermentation appears to contain certain unknown sulfur-containing compounds, in amounts up to 8.5 per cent of the total sulfur. This was shown by the liberation of inorganic sulfate on treatment of the filtered broth with bromine water (39).

Nitrogen Metabolism

No unusual amino acid has been recorded as being present in the mycelium of penicillin-producing strains (40, 41), nor any amino acid of unusual optical configuration (42). Amino acid synthesis by strain Q176 in a chemically defined medium containing ammonium salts as the sole nitrogen source has been studied by circular filter paper chromatography (43). A rapid synthesis occurred during the growth phase (1 to 4 days) with an appreciable accumulation of amino acids in the medium. In the phase of penicillin production (5 to 8 days) virtually all the amino acids disappeared from the medium, while there was no marked variation in the free amino acid content of the mycelium. The final stage (9 to 12 days) was characterized by a reappearance of amino acids in the medium, presumably owing to autolysis. Similar studies on corn-steep liquor cultures (44) and on both corn-steep liquor and chemically defined medium (41) have also been made. In the phase of penicillin formation restricted growth of the mycelium occurs, and hence the metabolism of amino acids taken up from the medium is of interest. Washed mycelium of strain Q176, harvested in the penicillin producing phase, can oxidize 23 different amino acids at varying rates (45). Further indication of an oxidative utilization is the presence in the mycelium of the same strain of an L-amino acid oxidase with a pH optimum of about 8 (46). However, this enzyme was produced only during active growth on a complex nitrogenous medium at a low pH. The fact that enzyme formation was largely suppressed when the mold was grown on a corn-steep liquor medium supplemented with ammonium sulfate, or grown on a chemically defined medium containing ammonium sulfate,

would indicate that ammonia was the preferred nitrogen source during active growth. For these reasons, a further role of this L-amino acid oxidase would appear to be the liberation of ammonia from amino acids in the phase of active growth. A D-amino acid oxidase has also been prepared from the mycelium of strain Q176 (47).

Glucose Metabolism

As well as utilizing glucose for energy and synthetic purposes, the penicillia also convert carbohydrate into mycelial structures and reserve polysaccharide (48) and under certain circumstances into products such as gluconic and other organic acids which accumulate extracellularly (19, 49).

Fragmentary evidence exists that the Embden-Meyerhof scheme is utilized for energy and synthetic purposes. Aldolase, triosephosphate isomerase and triosephosphate dehydrogenase are present in the mycelium of a strain of *P. notatum* (50), and a soluble, cytochrome-linked lactic dehydrogenase has been prepared from strain NRRL 1951-B25 (51). In addition, adenosine triphosphate, adenylic acid and mannose-1-phosphate have been isolated from the mycelium of strain Q176 (52). Some evidence has also been presented that an oxidative utilization of glucose may occur as well as the classical glycolytic pathway (53). Minced (*sic*) young mycelial cells of strain NRRL 1951-B25, were found to consume one molecule of oxygen per mole of C^{14}-glucose utilized, and radioactive glucose-6-phosphate and 6-phosphogluconate were identified by paper chromatography of an ethanol extract of the mycelium. Gluconic acid was consistently found, arising presumably by phosphatase action on 6-phosphogluconate. In this connection it may be mentioned that phosphatase activity has been found in the mycelium of strain NRRL 1951-B25 (51, 54). However, by whatever pathway glucose is catabolized, a considerable amount must be converted into acetate as shown by the extensive dilution of C^{14} labeled acetate during the second and third day of an eight-day culture of Q176 on a synthetic medium (55, 56).

It also seems probable that a tricarboxylic acid cycle operates in the penicillia. This fact may be inferred from radioactive studies (57), the preparation of cell free enzyme systems (51) and from a study of the metabolism of whole cells (56).

Carbon Dioxide Fixation

No growth of strain NRRL 1951-B25 occurred in a chemically defined medium in the absence of CO_2. Of many compounds tested, such as tricarboxylic acid intermediates and complex organic substances, only corn-steep liquor could obviate the requirement for CO_2. For maximum $C^{14}O_2$ fixation in a chemically defined medium, balanced proportions of metallic ions, glucose and sodium nitrate were found to be essential. After 15 minutes' exposure $C^{14}O_2$ was fixed into aspartate, glutamate and arginine of the mycelial protein (58). It is of interest that in the intact rat, these amino acids are the first to become labeled from assimilated $C^{14}O_2$ (59).

It has been suggested that CO_2 might be concerned in the biosynthesis of penicillin. Benzylpenicillin, isolated from a culture containing carboxyl-C^{14}-phenylacetic acid contained some C^{14} in the penicillamine portion and in the β-lactam carboxyl carbon, there being a rough correspondence between the average specific activity of the respiratory CO_2 and that of the β-lactam carboxyl carbon (60). In other experiments, however, no such incorporation of isotope has been observed from carboxyl-C^{14}-phenylacetic acid into parts of the benzylpenicillin molecule other than the acyl group (61), and no significant incorporation of isotope from $Na_2C^{14}O_3$ has been found (55).

PRECURSORS OF THE PENICILLIN MOLECULE

Many compounds have been tested for their ability to stimulate penicillin production. Increased penicillin yields may result from at least two alternative mechanisms. The substance added may act by a general stimulation of the mold's metabolism, or it may be a penicillin precursor whose synthesis by the mold is the rate-limiting step in the biosynthesis. For this reason, to demonstrate precursor function, it is necessary to show that the particular compound or a close derivative thereof is actually incorporated into the penicillin structure. This method has been used with marked success in the elucidation of the role of organic acids as precursors of the side-chain of penicillin.

Acyl side-chain. As already pointed out, penicillin is not a single chemical entity, but rather a family of closely related compounds differing only in the side-chain of the β-lactam-thiazolidine ring system. In a fermentation car-

ried out on a synthetic medium, the so-called natural penicillins are produced. These have been demonstrated by filter-paper chromatography to be Δ^2-pentenyl-penicillin, which is derived from $\beta\gamma$-hexenoic acid, *n*-heptylpenicillin from octanoic acid, *n*-amylpenicillin from hexanoic acid, *n*-butylpenicillin from *n*-valeric acid, *n*-propylpenicillin from *n*-butyric acid and traces of other penicillins of uncertain structure (62). It may be noted that a species of the fungus *Cephalosporium* produces a penicillin having D-α-aminoadipic acid as the R acid (63).

The relative proportions of these penicillins produced by the penicillia can be markedly altered by the addition of the appropriate acid to the fermentation. For example, added $\beta\gamma$-hexenoic acid raised the proportion of Δ^2-pentenylpenicillin formed from 47 per cent of the total penicillins in the control broth to 66 per cent of that in the experimental broth (62). Further, the addition of any one of a large number of certain organic acids results in the formation of a penicillin containing that acid in its side-chain. The proportion of these unnatural penicillins formed depends on certain conditions and these will be considered with particular reference to benzylpenicillin whose precursor is phenylacetic acid.

In the early work on penicillin it was realized that phenylacetic acid derivatives present in corn-steep liquor influenced the type of penicillin produced by the fungus and also markedly increased the total amount of penicillin formed (3, 4). This suggested a precursor function and conclusive proof of this was obtained by the addition of deuterophenylacetyl-DL-N^{15}-valine to a fermentation of strain NRRL 1976 in a corn-steep liquor medium. Deuterium analysis showed that 92.5 per cent of the benzylpenicillin isolated was derived from this precursor. In contrast, the low N^{15} content of the penicillin indicated that essentially only the phenylacetic group had been so utilized (64). Similar results have been obtained more recently using carboxyl-C^{13}-phenylacetic acid (65) and carboxyl-C^{14}-phenylacetic acid (60, 61). Phenylacetic acid is thus a true precursor of benzylpenicillin. Its effect on the type and total amount of penicillin produced is illustrated in table 2. It is to be noted in this table that some benzylpenicillin is produced in the unsupplemented medium. This is presumably due to the presence of phenylacetic acid derivatives in corn-steep liquor (66). The stimulation of penicillin production by addition of phenylacetic acid suggests strongly that it is the synthesis of the natural R group precursors which is the rate-limiting step of penicillin biosynthesis in an unsupplemented medium. The added precursor evidently largely displaces the small amount of naturally produced R group acids from the site of biosynthesis. Since the amount of the R group precursor is not now rate-limiting, penicillin production can in theory increase up to the level of the next rate-limiting reaction concerned in penicillin biosynthesis.

Many organic acids have been tested as precursors and some 40 unnatural penicillins have been biosynthesized in this way and characterized. In some cases it was found that they were formed to the virtual exclusion of the natural penicillins. Among the type of compounds found to be effective precursors were substituted mercaptoacetic, hydroxyacetic, polycyclic acetic, heterocyclic acetic and phenylacetic acids (3, 67–70), thiophenacetic acid (71) and mercaptoacetic acid (72). These results have led to several tentative conclusions relating structure to precursor function (3). Phenylacetic acid remained the best precursor, but the phenyl group could be substituted, or replaced by other ring systems, but not apparently by nitrogen-containing heterocycles. Benzoic acid derivatives did not appear to be utilized by the mold, and as only monosubstituted acetic acids were effective, it was concluded than an α-methylene group was es-

TABLE 2

The effect of phenylacetic acid on the quantity and type of penicillin produced in stirred aerated fermentations of strain Q176 in a corn-steep liquor medium

Amount of Phenylacetic Acid Added		Penicillin Yield	Penicillin Types (Per Cent of Total Yield)*				
Every 12 hr	Total		*n*-Heptyl-	*p*-Hydroxybenzyl-	Benzyl-	Δ^2-Pentenyl-	Amyl-
g/100 ml	*g/100 ml*	*units/ml*					
0	0	550	14.2	13.6	31.8	22.8	17.6
0.025	0.18	1321	2.7	2.1	82.8	7.3	5.1
0.050	0.40	1823	0.5	0.2	96.2	1.9	0.5

* Determined by filter paper chromatography. From the paper by Brown and Peterson (12).

sential. The suggestion was made that the efficiency of these compounds as precursors was due in part to their resistance to oxidation. It is to be noted that, in the absence of a ring system, compounds which contain an interrupting group, for example, a thioether linkage as in *n*-propylmercaptoacetic acid (67) or a double bond as in $\beta\gamma$-hexenoic acid, which would minimize β-oxidation, tend to be good precursors. β-Substituted acids are generally effective, as are $\beta\gamma$-unsaturated acids. $\alpha\beta$-Unsaturated acids are ineffective, although $\alpha\beta$-hexenoic acid has been reported to give rise to propylpenicillin (75). Acids which are α-substituted are generally not effective, although one exception is DL-α-methylbutyric acid (62). Substitution of the methyl group by an ethyl or larger group eliminates precursor activity and steric hindrance may thus be responsible for this effect (75).

It was realized, however, that only highly efficient precursors would stimulate the total yield of penicillin and give rise to appreciable amounts of new penicillins. Indeed, no detectable stimulation would be observed if the new penicillin were only a small fraction of the total amount formed or, in the case of a natural penicillin, if the mold already produced optimal amounts of that precursor. An apparent decrease in yield would even be observed if the new penicillin, formed in comparable amount, was less active on a molar basis than the aggregate of natural penicillins produced by the control (73).

With the use of the more sensitive technique of paper chromatography, many fatty acid derivatives have been shown to be precursors of the natural penicillins (62). Generally, free fatty acids, although increasing the percentage of a particular penicillin tend to decrease the total yield. In contrast, the corresponding triglycerides increase both the total amount and the percentage of that particular penicillin. For example, two biosynthetic penicillins, *n*-propylpenicillin and *n*-butylpenicillin were formed in good yield when tri-*n*-butyrin and tri-*n*-valerin were used as the respective precursors. Further evidence suggests that the mold is able to oxidize the higher fatty acids in the beta position and use the resulting smaller fragments as penicillin precursors. Thus, of the fatty acids containing an even number of carbon atoms, added tri-hexanoin increased the percentages of amyl- and propyl-penicillin in the resulting penicillin mixture, while added tri-octanoin increased the proportions of heptyl- and amyl-penicillins. Similar results were obtained with fatty acids containing odd numbers of carbon atoms, heptanoic and nonanoic acid both stimulating the production of *n*-butylpenicillin. β-Oxidation would also explain the observation that phenylbutyric acid derivatives give rise only to benzylpenicillin (74). Further, when hexanoic acid was added to a fermentation as much as 35 per cent appeared as a ketone, presumably β-ketohexanoic acid, within 12 hours (75). Fatty acid oxidation by *Penicillium chrysogenum* therefore appears to follow the classical scheme first outlined by Knoop. Such a mechanism of fatty acid oxidation had previously been shown to occur in many fungi (19).

The efficiency of these fatty acids as penicillin precursors is much less than that of phenylacetic acid. When equimolar amounts of either phenylacetic acid, valeric acid (as tri-valerin), or butyric acid (as tri-butyrin) were added to identical fermentations of synthetic media, the yields of penicillin were respectively, 280 units/ml (where 88.5 per cent of the total was benzylpenicillin), 148 units/ml (68 per cent butylpenicillin) and 92 units/ml (23.9 per cent propylpenicillin) (62). Since phenylacetic acid is metabolized at a much lower rate than are butyric, valeric, hexanoic or octanoic acids, (75), the available concentration of these acids at the site of penicillin biosynthesis would be lower. In addition, incomplete hydrolysis of the triglycerides of the fatty acids would also decrease the available amount of free acid.

The extent of precursor addition is limited by the fact that above certain critical levels penicillin yields decrease. This has been particularly noted with phenylacetic acid and depends on the pH, as well as the concentration of precursor present (13, 21, 28). It is believed that this effect is due to the toxicity of the undissociated molecule towards the fungal mycelium (21, 31). A similar toxic effect of free fatty acids has been observed (62). Maintenance of precursor levels is also limited by the appreciable metabolism of these compounds in ways other than for penicillin production (75), and large single additions of non-toxic derivatives of these precursors have therefore been used. It was supposed that these derivatives would be slowly converted to its actual precursor, so that concentration in the medium would be maintained despite simultaneous oxidation. In this manner, the triglycerides have been successfully

used as sources of the precursor fatty acids (62). Many derivatives of phenylacetic acid, such as esters, amides or substituted amines, phenylacetylated amino acids, β-phenylethylamine and phenylacetonitrile have also been tested, but in only a very few instances was the precursor efficiency comparable with that of an equivalent amount of phenylacetic acid (28). This may be partly attributed to the fact that although oxidation of phenylacetic acid by the mold is appreciable, some 30 per cent of a single non-toxic early addition to a fermentation remains in the final broth (75). Moreover, in many instances these derivatives are more toxic than phenylacetic acid at the same molar concentration (*e.g.*, β-phenylethylamine), or converted to the free acid at only a slow rate (*e.g.*, phenylacetylglycine), so that the concentration of the precursor is low (28). However, optimum yields of practically pure benzylpenicillin are obtained by the frequent addition of small amounts of phenylacetic acid over the fermentation period. Generally, 0.5–1.0 g/L every 12 hours for a stirred aerated fermentation (13) and 1.0–1.5 g/L every 12 hours for shake flasks (28) have given the best results.

At present time only benzylpenicillin appears to be produced commercially, and this is dictated by certain circumstances. Thus, it can be readily obtained as a nearly pure homogeneous sample directly from a fermentation. Moreover, the mold strain improvement programs have been consistently directed towards selecting strains which utilize phenylacetic acid most efficiently. Strain Q176 produces only 75 per cent benzylpenicillin at a precursor level of 500 mg/L per day, while the new pigmentless strains derived from Q176 under the same conditions produce essentially 100 per cent benzylpenicillin at only 200 mg phenylacetic acid per liter per day (14).

The incorporation of isotope from carboxyl-C^{14}-phenylacetic acid into benzylpenicillin by *P. chrysogenum* B-65 (Upjohn) grown in a corn-steep liquor medium has been studied (61). It is noteworthy that the molar radioactivity of the added phenylacetic acid (1530 counts/min/mmole) was appreciably higher than that of the penicillin isolated (1050 counts/min/mmole), while the phenylacetic acid remaining in the broth at the end of the fermentation was essentially unchanged in radioactivity (1470 counts/min/mmole) showing that the phenylacetic acid in the broth had not been appreciably diluted by non-radioactive material present in corn-steep liquor or by endogenous synthesis and extracellular excretion. On the other hand, intracellular phenylacetic acid appeared to be diluted by endogenous synthesis, as judged by the molar radioactivities of penicillin and phenylacetic acid, but it should be noted that no criteria of purity of the isolated penicillin are given. It is thus possible that the estimated radioactivity was low owing to the presence of impurities.

β-Lactam-thiazolidine ring system. Early studies on the precursors of the β-lactam-thiazolidine rings were based on the assumption that the addition of a precursor would increase the yield of penicillin. Although many compounds have been tested by this method, no conclusive results have been obtained (3, 4).

By analogy with the synthesis of cystathionine from cysteine and homoserine by the neurospora (76), it has been proposed that penicillin may be formed from L-cysteine and D-β-hydroxyvaline (77). However, neither cystine (78) nor cysteine and D-β-hydroxyvaline together (3) consistently stimulated penicillin production.

It is evident, however, that a precursor might fail to stimulate penicillin formation if it participated only in steps which are not rate-limiting. Recent work has therefore been carried out with the isotopic tracer technique, since a consideration of rate-limiting steps is not essential to the interpretation of results.

The utilization of possible sulfur-containing precursors has recently been studied by comparing the uptake of $Na_2S^{35}O_4$ into penicillin in their presence and absence. Although unlabeled sodium sulfate reduced the specific activity of penicillin by approximately 40 per cent, an equimolar amount of DL-methionine caused a 60 per cent and DL-cystine a 90 per cent reduction. Conversely, in experiments with L-S^{35}-cystine and unlabeled sodium sulfate, the preferential utilization of L-cystine sulfur for penicillin formation was confirmed. Neither D-cystine nor DL-penicillamine affected the uptake of $Na_2S^{35}O_4$ into penicillin (79). The equal incorporation of isotopic carbon, nitrogen and sulfur from L-β-C^{14}, N^{15},S^{35}-cystine into C_5, the side-chain nitrogen and the sulfur of penicillin has shown conclusively that the intact amino acid functions as a precursor. Since cystine of the mycelium had a similar isotope content to that of the penicillin, it was concluded that the observed dilution of isotopes

arose from endogenous synthesis of the amino acid. The poor utilization of D-β-C^{14}, N^{15},S^{35}-cystine in a similar fermentation indicates the stereochemical specificity of penicillin synthesis from cystine (80, 82).

The effect of methionine on the incorporation of $Na_2S^{35}O_4$ into penicillin (*cf.* 79) is probably due to its conversion into cystine. Methionine serves as a source of cystine sulfur both in the animal organism (83), and in the mold *Aspergillus nidulans* (84), so that the conversion of inorganic sulfate to penicillin may proceed as follows: sulfate → intermediates → methionine → cystine → penicillin.

The efficiency of incorporation of DL-β-C^{14}-serine and α-C^{14}-glycine and the distribution of isotope in the penicillin molecule (80, 81) are consistent with the metabolic pathway, glycine → serine → cystine → penicillin. C^{14}-formate has also been shown to be incorporated into penicillin although the position of the isotope was not determined (55). In animals, formate is a precursor of the β-carbon atom of serine (85) and this mechanism could account for the foregoing result.

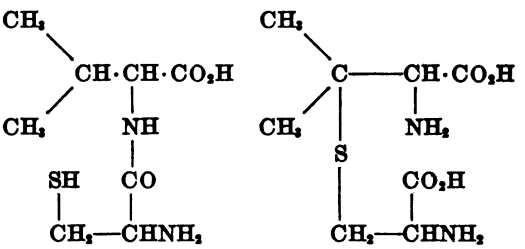
The available information concerning the origin of the penicillamine carbon atoms indicates that valine is a precursor of this portion of the penicillin molecule. Thus, DL-γ,γ'-C^{14}-valine (80, 81), DL-carboxyl-C^{14}-valine (86), DL-α-C^{14}-valine and L-U-C^{14}-valine (87), are all incorporated into the penicillamine moiety with dilutions of radioactivity which are similar to those found in the cystine experiments. Moreover, the distribution of radioactivity in the penicillin derived from DL-carboxyl-C^{14}- and L-U-C^{14}-valine indicates that the carbon chain of valine is utilized intact.

The source of the penicillamine nitrogen is, however, still unknown. In the original experiments with deutero-phenylacetyl-DL-N^{15}-valine (64), N^{15} was incorporated into penicillin with a far greater dilution than was deuterium, suggesting extensive loss of the valine nitrogen prior to its incorporation into penicillin. This low incorporation of the isotopic nitrogen might have been due, however, to extensive dilution of the amino acid by endogenous synthesis and to the presence of relatively large amounts of unlabeled valine in the corn-steep liquor medium. This latter explanation has been excluded by later experiments with C^{14},N^{15}-valine, where it has been found that the N^{15}:C^{14} ratio of the isolated penicillamine was markedly lower than that of the added valine. A similar change in the N^{15}:C^{14} ratio was observed in the valine of the mycelial protein indicating extensive transamination of this amino acid in the fermentation (87). The almost complete equilibration of valine nitrogen with the general nitrogen pool of the medium made it impossible to decide whether the intact valine molecule or only the carbon skeleton was used for penicillin synthesis. If the latter, the penicillamine nitrogen and the D-configuration of the asymmetric center at C_3 would be introduced into the penicillin structure at some later stage.

BIOSYNTHETIC MECHANISMS OF PENICILLIN FORMATION

It has thus been well established that the mold utilizes relatively simple compounds such as an organic acid, L-cysteine and valine or a close derivative for the formation of the unique penicillin structure. Since it is not likely that such a conversion would take place in one step, the mechanisms whereby these simple compounds are transformed into penicillin have been investigated by testing possible intermediates.

A primary stage in this conversion might be a condensation of L-cysteine with D-valine or a close derivative of this amino acid. This would lead to the formation of either a dipeptide, L-cysteinyl-D-valine (II), or a thioether β,β'-dimethyllanthionine (III).

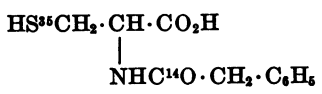


(II) (III)

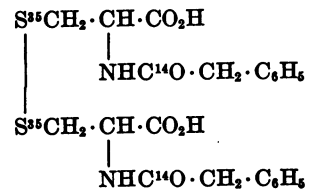
Compound III has been excluded as an intermediate on the grounds that it does not effectively reduce the incorporation of either S^{35}-labeled sulfate or S^{35}-labeled cystine into penicillin (88). This evidence, however, may not be entirely conclusive since the added thioether may not have been as freely available as inorganic sulfate or cysteine at the site of penicillin formation. *N*-phenylacetyl-L-cysteinyl-D-valine has been synthesized and was found to possess antibiotic

activity with a low degree of synergistic action when combined with benzylpenicillin (89). The possible precursor function of this compound has not, however, been determined.

Another possibility, the direct utilization of *N*-phenylacetyl-L-cysteine (IV) has also been studied, using *NN'*-dicarboxyl-C^{14}-phenylacetyl-L-S^{35}-cystine (V) which could presumably be reduced to the thiol compound (IV) in a fermentation. When this labeled compound



(IV)



(V)

was added to a penicillin fermentation, extensive hydrolysis and resynthesis occurred, and its intact utilization for penicillin biosynthesis could not be demonstrated. However, in the absence of added peptide no detectable amounts of *NN'*-diphenylacetylcystine were formed, suggesting that the phenylacetyl group is introduced into penicillin at another stage in the biosynthesis (90). The possibility has not been completely excluded, however, that phenylacetylcysteine is normally formed, but is not oxidized to the disulfide.

An alternative method of studying the mechanism of penicillin formation has been to grow the mold on a medium containing radioactive sulfate in order to identify possible sulfur-containing intermediates by paper chromatography of extracts of the mycelium. In this way several radioactive sulfur compounds were shown to be formed during a fermentation of strain Wis 51 to 20. However, the only compound which has been identified is choline sulfate (38), which is unlikely to be involved directly in the formation of penicillin. It is of interest that cyclic choline sulfate has also been isolated from *Aspergillus sydowi*, the dry mycelium containing as much as 0.26 per cent of this compound (91).

General cytoplasmic poisons such as phenol or mercuric chloride have been found to inhibit respiration and penicillin formation in washed mycelial suspensions to about the same extent. On the other hand, penicillin formation was specifically inhibited by 2:4-dinitrophenol at concentrations which had little effect on respiration, suggesting that oxidative phosphorylation may be important in penicillin formation (92). The analogous inhibition of amino acid incorporation into tissue protein by dinitrophenol *in vitro* (93) may indicate the similarity between penicillin formation and peptide bond synthesis in proteins. An even more specific inhibition of penicillin formation was observed with cyanide. For example, 2×10^{-5} M KCN had little effect on respiration but penicillin formation was depressed to about 25 per cent of normal (92). The low concentration of inhibitor required would suggest inactivation of a catalyst, presumably a metal-containing enzyme, and it is of interest to note in this connection that approximately 20 times as much iron is required for maximum penicillin production as for growth (31).

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